Time-to-turbidity model for non-proteolytic type B *Clostridium botulinum*[1]

Abstract

A model to predict the time for growth to turbidity from spores of non-proteolytic type B strains of *Clostridium botulinum* was developed in broth media with varying temperatures (4–28°C), pH values (5–7), NaCl additions (0–4%) and total spores (10^1–10^5). The model estimates the probability that a sample will have growth on a given day for up to 90 days of storage. The parameters of the model include the probability of growth after 90 days (P_{max}) and the mean time of growth (τ) for those tubes that showed growth. The 95% confidence interval ($CI_{95\%}$) for τ was also determined. The τ decreased with increasing temperature and pH, but NaCl levels below 3% had little effect. Decreasing the number of spores in a sample increased both τ and the confidence intervals about τ, reflecting the increasing uncertainty about the estimation of growth times for low spore numbers. © 1997 Elsevier Science B.V.

Keywords: Microbial pathogens; Risk assessment; Predictive microbiology

1. Introduction

Non-proteolytic strains of *Clostridium botulinum* have different growth characteristics than proteolytic strains, which affect the probability of their growth and toxin formation in foods (Simunovic et al., 1985; Kim and Foegeding, 1993; Lücke and Roberts, 1993; Lund and Notermans, 1993). Their ability to survive mild heating and grow at refrigeration temperatures makes them a potential hazard in minimally processed, refrigerated, ready-to-eat foods. Considerable research has been performed on the non-proteolytic type E strains that are primarily found in marine foods. Non-proteolytic type B strains have also been isolated from marine sources in North America and are very common in Europe

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[1] Mention of brand or firm name does not constitute an endorsement by the US Department of Agriculture above any similar names not mentioned.

(Simunovic et al., 1985). In considering the risks from non-proteolytic type B strains in North America, Simunovic et al. (1985) state "the possibility of their presence should not be disregarded, since large-scale food production could provide a volume and medium necessary for the inclusion and outgrowth of a rarely occurring organism when the conditions are favorable. Also, the possibility of contamination of the product from imported meats, spices or other ingredients should not be overlooked." Since 1950, three cases of non-proteolytic type B toxicity have occurred in the US (Hatheway, 1993) and two in Canada (Hauschild, 1993).

Modeling of *C. botulinum* growth and toxin formation was reviewed by Baker and Genigeorgis (1993). Modeling of non-proteolytic strains has been mostly completed for type E strains (Baker et al., 1990) or mixtures of types E, B and/or F (Lindroth and Genigeorgis, 1986; Ikawa and Genigeorgis, 1987; Genigeorgis et al., 1991; Meng and Genigeorgis, 1993). Lund et al. (1990) presented a probability of growth model for a four-strain mixture of non-proteolytic B vegetative cells with temperature (6–30°C), pH (4.8–7.0) and sorbic acid concentrations (0–2.27 g/l). Later, the temperature effect on growth rates of the B strain vegetative cells were modeled with the 'square root model' over the range of 4–35°C (Graham and Lund, 1993). Jensen et al. (1987) modeled the probability of growth of two non-proteolytic B strain spores and vegetative cells for 28 days in broths as affected by inoculum size and temperature. The probability of growth from a single spore was determined using serial dilutions and MPN tables. A regression equation described the probability during storage with temperature for a pooled type E, F and non-proteolytic B inoculum. Graham et al. (1996) developed a growth model from 10^5 type B, E and F spores with factors of temperature, pH and NaCl.

A logistic model for the time-to-growth (turbidity) of proteolytic spores of *C. botulinum* was developed that had parameters for maximum probability of a sample becoming turbid, the rate samples became turbid, and the mean time for the growth to occur (Whiting and Call, 1993). The environmental factors modeled were temperature, pH and NaCl levels. Spore numbers were fixed at 10^4/sample.

This paper presents a model for the time-to-turbidity of non-proteolytic type B spores in broths with varying temperatures (5–28°C), pH values (5–7), added NaCl (0–4%) and spore numbers (10^1–10^5/sample) for up to 90 days. The inclusion of spore numbers and the calculation of confidence ranges for the time parameter are significant advances over the version of the model reported for proteolytic strains (Whiting and Call, 1993).

2. Materials and methods

A mixture of six *C. botulinum* non-proteolytic B strains was used (D8B from ERRC USDA, Philadelphia, PA; KAP8 and ATCC 7844 from National Food Processors, Washington, DC; 2129, CBW25 and 17B from Campbell Soup, Camden, NJ). Strains were individually grown in 500 ml botulinal assay media (BAM) (Huhtanen, 1975) at 37°C for 3 weeks inside an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI). They were centrifuged (15 min at $5860 \times g$) and resuspended in sterile water. The spore populations of each strain were determined by heat shocking aliquots at 55°C for 10 min and plating onto BAM agar inside the anaerobic chamber using a Spiral Plater (Spiral Systems, Bethesda, MD). The plates were incubated inside the chamber at 37°C for 1.5–2 days and visually counted. A spore mixture containing equal numbers of each strain was made which had a total of 10^7 spores/ml. The spore mixture was stored in a refrigerator (6°C). Confirmation of numbers and purity was performed by incubating aerobic and anerobic plates of the heat-shocked spore mixtures. Only anaerobic growth of Gram-positive, catalase negative rods was observed.

To inoculate broth tubes, an aliquot (1.0 ml) of the spore mixture was heat-shocked as above and dilutions of 10^6, 10^5, $10^{3.5}$ and 10^2 spores/ml were made using sterile water. When 0.10 ml of these dilutions were inoculated into 10 ml of broth, the respective total number of spores per tube was 10^5, 10^4, $10^{2.5}$ and 10^1.

BAM broth was prepared without thioglycollate and adjusted to pH 5–7 using 0.1 N HCl and 0–4% NaCl (w/v) was added. Seven combinations of pH and NaCl were made and 10 ml of each were dispensed into culture tubes. After autoclaving, the tubes were cooled overnight inside the anaerobic chamber. The broths were inoculated with 0.10 ml of one of the spore mixture dilutions and covered with 1–2 ml VASPAR (petroleum jelly-paraffin, sterilized). After solidification of the VASPAR, the tubes were removed from the anaerobic chamber and placed in aerobic incubators at 4, 8, 12, 19, or 28°C. There were 35 combinations of temperature × pH × % NaCl, and with the different inocula levels the total number of treatments was 103. The number of tubes for a treatment combination ranged from five, for combinations of rapid (1–2 day) and nearly total growth ($P_{max} > 0.9$), to 30 tubes for combinations with slow and partial growth (low P_{max}). The model was built on observing a total of 1635 tubes, in addition to numerous uninoculated control tubes, to confirm that sterile handling was achieved and for visual comparisons during storage of inoculated tubes. The entire experiment was separated into three starting times in a fractional factorial design.

Tubes were observed daily for the first 2 weeks and then three times per week for the remaining 90 days. The time for formation of haze, sediment or gas was noted. Presumptive positive tubes were kept in the incubator for several additional observations before discarding.

Modeling followed the procedure of Whiting and Call (1993). For each of the 103 treatment combinations, the fraction of positive tubes at each observation time was calculated. The general observed pattern was a time period without any turbid tubes, then a period with increasing numbers of positive tubes, followed by no increase for the remaining storage period. The sigmoidal increase in positive tubes, with respect to time, was fitted to a logistic equation using SigmaPlot 4.0 (Jandel Scientific, Corte Madera, CA) to determine the parameter values. The logistic equation used was

$$P = P_{max}/(1 + \exp^{k(\tau - t)})$$

This probability (P) at a time (t, days) equation has parameters for the maximum fraction of positive tubes (P_{max}), time for midpoint of the curve (τ days) and rate of the increase in positive tubes (k/days).

The parameters (P_{max}, k, τ) for the 103 combinations of temperature-pH-NaCl-spore inoculum were then subjected to polynomial regression analysis (SAS Institute, Cary, NC). For calculating the τ parameter regression equation, the 31 combinations that showed no turbidities ($P_{max} = 0.00$ at 90 days) were removed and the $\log_{10}$ τ fitted. The 95% confidence intervals ($CI_{95\%}$) for τ were also calculated (Draper and Smith, 1989). The resulting regression equation will calculate negative values for certain combinations, but these are considered to signify no growth. Similarly for P_{max} and k, calculations of less than zero are interpreted as areas of no growth and a P_{max} calculation greater than 1.0 is interpreted as growth in all tubes.

3. Results and discussion

Representative data sets from three treatment combinations are illustrated in Fig. 1. They show three different τs, two with P_{max} equal to 0.67 and

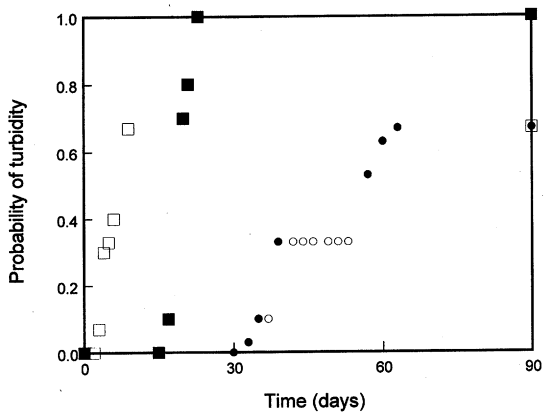

Fig. 1. Increasing probability of turbidity for three treatment combinations with time. The conditions are 28°C, pH 5.0, 0% NaCl, 10^4 spores (□); 12°C, pH 7.0, 0% NaCl, $10^{2.5}$ spores (■); 4°C, pH 5.0, 0% NaCl, 10^4 spores (●○). For the latter condition, the curve fitting used data represented by the solid circles only.

Table 1
Treatment combinations, parameter values of data fits to logistic model and parameter values calculated from the regression equations

Factors				Tubes (n)	Parameter values fitted to individual observed curves			Values calculated by regression equations				
Temp (°C)	pH	NaCl (%)	Inoculation (log)		P_{max}	k (days^{-1})	τ (days)	P_{max}	k (days^{-1})	τ (days)	LCLτ (days)	UCLτ (days)
4	7.0	0	1.0	10	0.00	0.0		0.19	18.7	—[a]	35.4	—[a]
4	7.0	0	2.5	10	0.00	0.0		0.33	18.7	60.3	30.6	—[a]
4	7.0	0	4.0	15	0.73	1.3	17	0.69	18.7	25.3	15.9	40.7
4	7.0	0	5.0	5	1.00	20.4	18	1.06	18.7	11.6	6.6	20.5
4	7.0	4	1.0	10	0.00	0.0		−0.24	−18.7	—[a]	—[a]	—[a]
4	7.0	4	2.5	10	0.00	0.0		0.04	−7.9	—[a]	73.4	—[a]
4	7.0	4	4.0	15	0.20	0.6	35	0.55	2.9	55.3	30.6	—[a]
4	6.0	0	1.0	10	0.00	0.0		−0.02	4.9	—[a]	54.7	—[a]
4	6.0	0	2.5	10	0.00	0.0		0.21	4.9	83.2	43.9	—[a]
4	6.0	0	4.0	15	0.69	0.2	21	0.66	4.9	29.7	19.9	44.7
4	6.0	0	5.0	5	1.00	13.9	12	1.09	4.9	12.2	7.6	19.7
4	6.0	2	1.0	20	0.00	0.0		−0.20	−10.4	—[a]	58.4	—[a]
4	6.0	2	2.5	20	0.00	0.0		−0.10	−4.9	76.3	41.0	—[a]
4	6.0	2	4.0	30	0.68	0.2	30	0.63	0.5	23.1	15.7	34.5
4	5.5	1	1.0	20	0.00	0.0		−0.20	−4.5	—[a]	75.6	—[a]
4	5.5	1	2.5	20	0.00	0.0		0.10	−1.8	—[a]	56.3	—[a]
4	5.5	1	4.0	30	0.70	0.1	34	0.64	0.9	32.2	22.5	46.4
4	5.0	0	1.0	20	0.00	0.0		−0.23	2.4	—[a]	—[a]	—[a]
4	5.0	0	2.5	20	0.00	0.0		0.09	2.4	—[a]	—[a]	—[a]
4	5.0	0	4.0	30	0.62	0.5	39	0.63	2.4	74.5	47.1	—[a]
4	5.0	0	5.0	10	1.05	2.2	34	1.12	2.4	27.4	16.5	45.6
4	5.0	3	1.0	20	0.00	0.0		−0.44	−15.4	—[a]	—[a]	—[a]
4	5.0	3	2.5	20	0.00	0.0		−0.02	7.3	—[a]	84.5	—[a]
4	5.0	3	4.0	30	0.00	0.0		0.63	0.8	58.5	29.9	—[a]
8	7.0	0	4.0	5	1.00	23.4	9	0.82	25.5	10.0	7.0	14.4
8	7.0	4	4.0	5	1.00	23.4	11	0.67	12.9	21.9	14.0	34.5
8	6.0	0	4.0	5	1.00	23.4	11	0.75	10.4	12.8	9.4	17.6
8	6.0	2	4.0	10	1.00	11.4	9	0.71	7.5	10.0	7.5	13.5
8	5.5	1	4.0	10	1.00	23.4	11	0.70	6.5	14.5	11.3	18.8
8	5.0	0	4.0	10	1.00	6.3	26	0.68	6.5	35.2	25.0	49.8
8	5.0	3	4.0	10	1.00	13.9	28	0.68	7.3	27.7	16.5	47.1
12	7.0	0	1.0	10	1.00	18.7	20	0.61	30.6	12.4	6.8	22.9
12	7.0	0	2.5	10	1.01	94.1	19	0.65	30.3	9.0	6.1	13.3
12	7.0	0	4.0	15	0.99	0.6	9	0.92	30.6	4.5	3.2	6.3
12	7.0	4	1.0	10	0.00	0.0		0.18	−0.6	52.6	20.1	—[a]
12	7.0	4	2.5	10	0.00	0.0		0.36	10.2	27.5	15.6	48.8
12	7.0	4	4.0	15	0.67	2.5	12	0.78	21.0	9.9	6.7	14.7
12	6.0	0	1.0	10	0.00	0.0		0.32	14.1	24.2	12.9	45.7
12	6.0	0	2.5	10	0.20	15.8	45	0.45	14.1	14.9	10.3	21.6
12	6.0	0	4.0	15	0.93	1.4	10	0.81	14.1	6.3	4.6	8.6
12	6.0	2	1.0	20	0.05	0.9	23	0.14	1.9	26.3	14.1	49.4
12	6.0	2	2.5	20	0.30	0.2	20	0.35	7.3	13.7	9.9	19.1

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Temp (°C)	pH	NaCl (%)	Inoculation (log)		P_{max}	k (days^{-1})	τ (days)	P_{max}	k (days^{-1})	τ (days)	LCLτ (days)	UCLτ (days)
12	6.0	2	4.0	30	0.64	1.5	10	0.78	12.8	4.9	3.7	6.6
12	5.5	1	1.0	20	0.00	0.0		0.10	4.8	39.9	20.1	79.8
12	5.5	1	2.5	20	0.00	0.0		0.31	7.5	20.8	15.2	28.9
12	5.5	1	4.0	30	0.66	1.5	12	0.75	10.2	7.5	5.9	9.6
12	5.0	0	1.0	20	0.00	0.0		0.03	8.8	—[a]	40.1	—[a]
12	5.0	0	2.5	20	0.10	6.2	57	0.26	8.8	52.7	32.6	85.8
12	5.0	0	4.0	30	0.66	0.8	35	0.70	8.8	19.0	13.9	26.1
12	5.0	3	1.0	20	0.00	0.0		−0.18	−4.3	—[a]	36.7	—[a]
12	5.0	3	2.5	20	0.00	0.0		0.15	3.8	53.4	25.8	—[a]
12	5.0	3	4.0	30	0.72	0.1	38	0.71	12.0	15.0	9.6	23.7
15	7.0	0	4.0	5	0.80	39.1	2	0.99	33.2	2.7	1.9	3.8
15	7.0	4	4.0	5	1.00	1.4	6	0.85	26.0	5.9	4.1	8.8
15	6.0	0	4.0	5	1.00	22.6	3	0.85	15.7	4.0	2.9	5.6
15	6.0	2	4.0	10	1.00	39.5	4	0.82	15.5	3.2	2.3	4.3
15	5.5	1	4.0	10	1.00	39.5	4	0.78	11.9	5.0	3.9	6.5
15	5.0	0	4.0	10	0.90	23.0	9	0.71	9.4	13.0	9.5	18.0
15	5.0	3	4.0	10	0.99	1.3	10	0.72	14.3	10.4	6.7	16.1
19	7.0	0	1.0	10	1.00	93.9	2	0.92	35.1	3.1	1.9	5.1
19	7.0	0	2.5	10	1.00	93.9	2	0.88	35.1	2.6	1.9	3.7
19	7.0	0	4.0	15	0.67	25.3	1	1.07	35.1	1.5	1.0	2.3
19	7.0	4	1.0	10	0.00	0.0		0.48	9.3	13.4	6.3	28.7
19	7.0	4	2.5	10	0.80	1.6	22	0.59	20.2	8.1	5.3	12.7
19	7.0	4	4.0	15	0.93	9.0	3	0.93	31.0	3.4	2.2	5.8
19	6.0	0	1.0	10	0.61	1.7	4	0.56	16.2	7.2	4.6	11.3
19	6.0	0	2.5	10	1.00	23.1	3	0.61	16.2	5.1	3.7	7.2
19	6.0	0	4.0	15	1.00	23.6	2	0.89	16.2	2.5	1.8	3.5
19	6.0	2	1.0	20	0.30	8.4	5	0.38	6.8	7.8	5.1	12.1
19	6.0	2	2.5	20	0.75	39.0	3	0.50	12.2	4.7	3.6	6.4
19	6.0	2	4.0	30	1.00	2.1	2	0.86	17.6	2.0	1.4	2.7
19	5.5	1	1.0	20	0.00	0.0		0.30	7.1	12.8	8.0	20.7
19	5.5	1	2.5	20	0.15	0.5	5	0.43	9.9	7.8	6.0	10.3
19	5.5	1	4.0	30	0.86	5.4	3	0.79	12.6	3.3	2.5	4.3
19	5.0	0	1.0	20	0.00	0.0		0.20	8.6	35.1	17.5	20.7
19	5.0	0	2.5	20	0.00	0.0		0.34	8.6	21.3	14.3	31.8
19	5.0	0	4.0	30	0.68	0.2	5	0.71	8.6	8.9	6.5	12.3
19	5.0	0	5.0	10	0.70	3.6	4	1.08	8.6	4.0	2.5	6.5
19	5.0	3	1.0	20	0.00	0.0		0.00	−0.4	45.7	16.2	—[a]
19	5.0	3	2.5	20	0.00	0.0		0.24	7.7	21.7	11.7	40.4
19	5.0	3	4.0	30	0.67	24.3	5	0.71	15.8	7.1	4.6	11.1
28	7.0	0	1.0	10	1.00	20.5	1	1.24	32.8	1.0	0.6	1.7
28	7.0	0	2.5	10	1.00	20.5	1	1.09	32.8	1.0	0.6	1.6
28	7.0	0	4.0	15	1.05	1.6	1	1.18	32.8	0.7	0.4	1.3

Table 1
Treatment combinations, parameter values of data fits to logistic model and parameter values calculated from the regression equations

Factors				Tubes (n)	Parameter values fitted to individual observed curves			Values calculated by regression equations				
Temp (°C)	pH	NaCl (%)	Inoculation (log)		P_{max}	k (days^{-1})	τ (days)	P_{max}	k (days^{-1})	τ (days)	LCLτ (days)	UCLτ (days)
28	7.0	4	1.0	10	1.01	2.3	4	0.80	14.1	4.2	1.5	11.6
28	7.0	4	2.5	10	1.00	24.2	3	0.80	25.0	3.1	1.9	5.1
28	7.0	4	4.0	15	0.93	96.6	2	1.03	35.8	1.6	0.8	3.1
28	6.0	0	1.0	10	0.93	1.2	4	0.79	10.9	2.7	1.7	4.4
28	6.0	0	2.5	10	0.99	1.2	2	0.74	10.9	2.4	1.6	3.5
28	6.0	0	4.0	15	0.68	1.7	2	0.91	10.9	1.4	0.9	2.2
28	6.0	2	1.0	20	0.96	0.9	3	0.61	5.0	3.0	2.0	4.6
28	6.0	2	2.5	20	0.99	1.8	2	0.63	10.4	2.2	1.6	3.2
28	6.0	2	4.0	30	0.66	2.9	1	0.87	15.9	1.1	0.7	1.7
28	5.5	1	1.0	20	0.26	1.3	4	0.49	2.1	5.5	3.6	8.4
28	5.5	1	2.5	20	0.50	23.5	3	0.51	4.8	4.0	2.9	5.6
28	5.5	1	4.0	30	1.00	23.6	2	0.77	7.5	2.0	1.5	2.9
28	5.0	0	1.0	20	0.55	2.6	26	0.34	0.2	16.5	9.0	30.3
28	5.0	0	2.5	20	0.50	10.2	27	0.38	0.2	12.1	7.8	19.0
28	5.0	0	4.0	30	0.68	0.7	5	0.64	0.2	6.1	4.0	9.5
28	5.0	0	5.0	10	0.91	2.8	4	0.94	0.2	3.2	1.7	5.8
28	5.0	3	1.0	20	0.00	0.0		0.13	−3.5	26.6	8.5	55.3
28	5.0	3	2.5	20	0.00	0.0		0.28	4.6	12.5	6.4	24.4
28	5.0	3	4.0	30	0.67	22.2	3	0.64	12.7	4.9	2.7	9.1

[a] Value exceeds 90 days.

Table 2
Regression equations and statistics for P_{max}, k and τ

k	$158.45 - 60.37\ pH + 0.3397\ Temp \times pH + 0.1954\ Temp \times NaCl - 1.708\ pH \times NaCl + 1.808\ NaCl \times Inoc - 0.055588\ Temp^2 + 5.596\ pH^2$
r^2	0.32
n	103
P_{max}	$-1.636 + 0.2274\ pH + 0.3578\ Inoc + 0.009878\ Temp \times pH - 0.007804\ Temp \times Inoc - 0.01892\ pH \times NaCl - 0.05891\ pH \times Inoc + 0.02416\ NaCl \times Inoc - 0.000554\ Temp^2 + 0.05072\ Inoc^2$
r^2	0.73
n	103
$\log_{10}\tau$	$11.064 - 0.07847\ Temp - 2.362\ pH - 0.2667\ NaCl - 0.3716\ Inoc - 0.009911\ Temp \times pH + 0.000154\ Temp \times NaCl + 0.006175\ Temp \times Inoc + 0.03344\ pH \times NaCl + 0.04713\ pH \times Inoc - 0.02362\ NaCl \times Inoc + 0.001816\ Temp^2 + 0.1649\ pH^2 + 0.0528\ NaCl^2 - 0.03601\ Inoc^2$
r^2	0.86
n	72

Inoc, log of the inoculation size.

one with a much slower rate of tubes becoming positive (k). This latter data set (4°C, pH 5, 0% NaCl, 10^4 spores) contains many observations where the number of positive tubes did not increase from the previous observation. They are indicated with open symbols. These points were not used in the model fitting which make the τ value slightly smaller than if all observations were used. This means that the model will predict growth sooner and provides a conservative bias to the prediction. For fitting the logistic equation, observation times with no positive tubes ($P = 0$) and times at the maximum probability (P_{max}) were eliminated, except for the first and last observations of each phase. This avoided having an excessive number of times where $P = 0.0$ or P_{max} in the equation fitting process. To obtain the closet fit through the changing values of P, the fitting process was not bounded at $0 \leq P \leq 1$.

The parameter values of the fitted logistic model for each of the 103 treatment combinations are given in Table 1. Where no tubes turned turbid, P_{max} and k are zero and the τ is blank. The fits of the treatments where growth occurred within a 1–2 day period resulted in a large but imprecise estimate for k because of the few time-probability points available to fit. Best estimates were made for these fits. After stepwise backwards elimination of terms with significance levels greater than 0.2 whose removal does not reduce the r^2 more than 0.02, the regression equation for k was significant ($P < 0.01$) but the r^2 was only 0.32 and F was 6.4 (Table 2). The practical effect is that at favorable conditions the growth rate is as fast as the precision of this model, 1–2 days. Overall, the k parameter is not the major parameter in determining the predictions or their interpretation. This was observed previously with the proteolytic *C. botulinum* model (Whiting and Call, 1993). The P_{max} regression equation was also simplified by backwards elimination ($P < 0.001$, $F = 27.7$, $r^2 = 0.73$). Of the 73 treatment combinations having growth, 32 had P_{max} values greater than 0.1 and less than 0.9. The time that turbidity occurred (τ) is probably the most important parameter in this model. The variance of τ increased with increasing values for τ, therefore, the regression for the $\log_{10}\tau$ was calculated ($P < 0.001$, $F = 24.4$, $r^2 = 0.86$). The relation between times-to-turbidity and spore numbers is illustrated with estimates generated by the regression equation at 21°C, 3% NaCl and pH 6.0 (Fig. 2). Also indicated are the lower and upper $CI_{95\%}$s about the τ estimates. With high numbers of spores (10^4–10^5), turbidity is expected within 1–2 days and the CIs are small. With only 100 spores, the estimate is 6.5 days and the $CI_{95\%}$ is 4.3 and 9.9 days. The expansion of the CIs is a characteristic of regression when variables go toward the extremes of their range. However, the variance

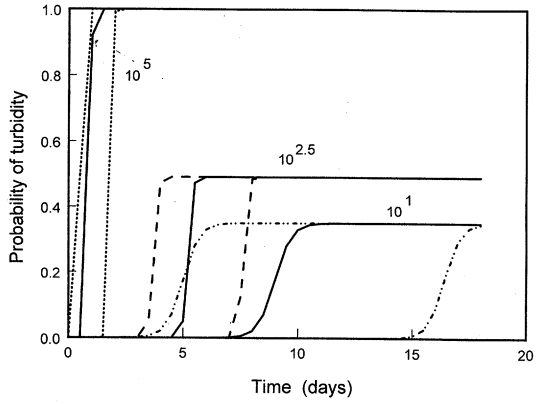

Fig. 2. Generated probability curves and $CI_{95\%}$ for the τ values for three spore numbers. Conditions are: 21°C, pH 6.0, 3.0% NaCl with 10^1, $10^{2.5}$ and 10^5 spores. Solid lines represent the estimated curve and the dotted lines the curves at the lower and upper CIs of τ.

about τ increased as τ increased. Therefore, the inverse log of the regressions CI has a larger range at higher τ values. For spore numbers, part of the increased variance can be rationalized by the spore population having a distribution of germination times. The larger the population, the more likely at least one spore with a shorter germination time will be present to initiate growth.

The influence of spore numbers is also shown in Fig. 3 with the generated curves for three spore loads and their $CI_{95\%}$s about τ. As spore numbers

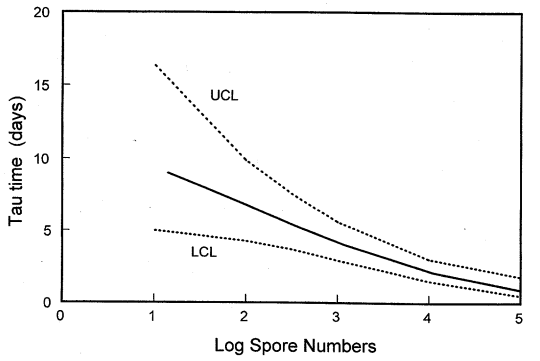

Fig. 3. Generated estimates of τ and CIs with various spore numbers. The other parameters values are 21°C, pH 6.0, 3.0% NaCl.

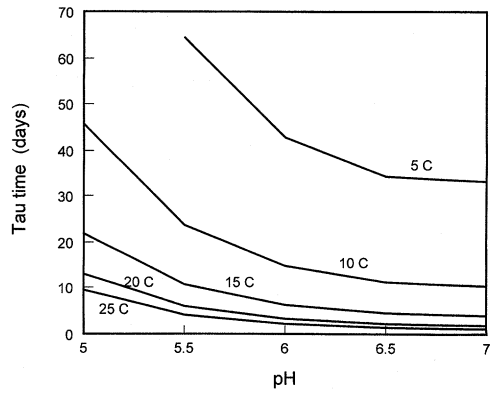

Fig. 4. Interaction of temperature and pH on the τ values. The generated values are for 0.5% NaCl and 10^3 spores.

decrease, the probability of any tube becoming turbid (P_{max}) decreases and the time for turbidity (τ) increases. However, consideration of the confidence intervals lessens the effect of spore numbers. The estimated growth times at the lower CI, when the number of spores decreases from $10^{2.5}$ to 10^1, only increased from 4 to 5 days while the τ increases from 5–9 days. The effect of spore numbers needs to be considered when designing inoculated pack studies because packages with 10^4 spores will undoubtedly become toxic sooner and perhaps more consistently (P_{max}) than foods with lower numbers of spores. However, this is balanced by the need to extrapolate from the relative few laboratory samples to the large number of packages in a commercial production run. It would be more prudent to design a food using the lower confidence interval than the estimated mean value (τ). This demonstrates the need to have an estimate of the variation when interpreting food microbiology data.

Temperature and pH have a major impact on the time to turbidity, as shown by the data generated from the equations (Fig. 4). Low temperatures and pH values increase the times. Growth eventually occurs at refrigeration temperatures at higher pH values. Fig. 4 is based upon 10^3 spores and the previous paragraphs indicate how this may change with different spore numbers, or if the lower CIs were determined. The influence of NaCl is not as pronounced as the other factors

(Fig. 5). The times for turbidity are not greatly affected with less than 3% NaCl, but increase with 4% NaCl.

The estimates of τ and the $CI_{95\%}$ ranges from this model are compared to times for growth or toxin reported in the literature (Table 3). In many cases, they had to be estimated or inferred from the reported data. For models based on the probability of a single spore growing, the time to reach log P equal to -2 was selected for comparison. When doubling times were reported (Graham and Lund, 1993), 15 doublings were considered to result in observable growth or toxin formation. The type B strain broth data for Soloman et al. (1982); Jensen et al. (1987); Graham and Lund (1993); and Peck et al. (1995) agree well with this model.

The strain grown in cooked meat medium by Eklund et al. (1967) had longer times to gas formation (21 days) and toxin development (27 days) than the times calculated at the closest pH and spore numbers allowed by this model. The studies using fish or poultry meats frequently reported somewhat shorter times, particularly at the lower temperatures or lower spore numbers. However, these studies used strain mixtures, and Fig. 2 in Jensen et al. (1987) indicated that type E strains outgrow type B. In Ikawa and Genigeorgis (1987) some toxin samples were typed and only type B toxin was found, although in a similar study, both B and E toxins were observed (Genigeorgis et al., 1991). Studies with other pathogens have indicated that differences between strains in growth and survival times can be large, often two or threefold (Shah et al., 1991; Barbosa et al., 1994). The differences between this study and the literature data can be a consequence of the different strains used in the various studies.

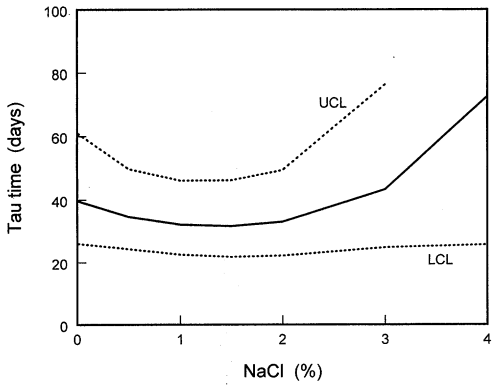

Fig. 5. The effect of NaCl concentration on the τ values and CIs. The generated values are for 7°C, pH 5.7, 10^4 spores.

The recent model by Graham et al. (1996) estimated the growth curve from 10^5 total spores in 100 ml, comparisons with this model having 10^4 total spores in 10 ml showed similar estimations. These spore numbers were chosen for comparison to have a comparable number of spores and number of cell doublings to turbidity. The Graham model provided estimates for both lag and doubling times, where the model proposed in this paper included both in the time-to-turbidity. However, this paper's model had spore numbers as a variable, recognizing that at low spore numbers and a less favorable environment, the probability distribution of spore germination and outgrowth became an important factor in estimating the likelihood of growth and toxin formation. At the low temperature environmental condition (Table 3, Graham et al., 1996) of 5.0°C, pH 6.0 and 0.1% NaCl, a lag time of 304 h and a doubling time of 29 h (Baranyi model) was reported. Estimated times to turbidity from 10^2, 10^3 and 10^4 spores/ml were 29.6, 24.8 and 21.1 days, respectively, assuming turbidity at 10^6 cells/ml and 7, 10 and 14 doublings would be necessary. The regression equations in this paper estimated 45.9, 23.2 and 9.6 days to turbidity for 10^3, 10^4 and 10^5 total spores (10^2, 10^3 and 10^4 spores/ml in the test conditions). At the 10^3 spores/ml the models were equivalent, however, this paper's model indicated a major effect of spore numbers. At more favorable conditions of 20°C, pH 6.9 and 3% NaCl the estimate of time-to-turbidity by the Graham model was 1.6 days and the estimates for 10^3, 10^4 and 10^5 total spores by the equations in this paper were closer together at 3.1, 1.7 and 0.8 days, respectively. The ideal model for spore forming microorganisms would include the probability of spore germination/outgrowth with an estimated value and variation for the growth rate or doubling time.

Table 3
Comparison of estimates by this model to literature values

Medium and type	Temp (°C)	pH	Salt (%)	Spores (log no.)	Reported time (days)	Model estimates (days)		Ref.
						Time	Confidence range	
Cooked meat medium, B	5.6	7.2[a] (7.0)	0.5	5.3[a] (5.0)	21	6.9	3.9–12.6	Eklund et al., 1967
Broth B	8	(6.0)	(0.5)	4	10–15	11.0	8.7–14.0	Soloman et al., 1982
	12	(6.0)	(0.5)	4	3–6	5.4	4.3–6.9	
	26	(6.0)	(0.5)	4	<3	1.3	1.0–1.8	
Fish	8	6.5	(0.5)	4	12	9.0	7.0–11.6	Ikawa and
B, E and F	17	6.5	(0.5)	2	3	4.7	3.6–6.3	Genigeorgis, 1987
B toxin only	30	6.5	(0.5)	2	1	1.2	0.8–1.9	
Fish	4	(6.5)	(0.5)	3	21	44	26.9–72	Lindroth and
B, E and F	8	(6.5)	(0.5)	4	12	9	7.0–11.6	Genigeorgis, 1986
No toxin typing	8	(6.5)	(0.5)	2	15	28	17.4–44.2	
	12	(6.5)	(0.5)	1	6	15	9.0–25.3	
	17	(6.5)	(0.5)	2	3	4.7	3.6–6.3	
	30	(6.5)	(0.5)	2	1	1.2	0.8–1.9	
Broth, B	12	7.4[a] (7.0)	(0.5)	2	7	9.8	6.1–16.0	Jensen et al., 1987
	16	7.4[a] (7.0)	(0.5)	2	<4	4.5	2.9–7.0	
	20	7.4[a] (7.0)	(0.5)	2	<3	2.4	1.5–3.7	
Turkey	16	6.2	(0.5)	1	2.5	8.7	5.8–13.2	Genigeorgis et al., 1991
B and E	16	6.2	(0.5)	4	1.5	2.7	2.1–3.5	
Both toxins	16	6.2	2.2	1	9	11.1	6.8–18.3	
	16	6.2	2.2	4	2.5	2.6	1.9–3.6	
	8	6.2	2.2	1	14	45.6	22.3–95.4	
	8	6.2	2.2	4	8	9.9	7.8–12.6	

Table 3
Comparison of estimates by this model to literature values

Medium and type	Temp (°C)	pH	Salt (%)	Spores (log no.)	Reported time (days)	Model estimates (days)		Ref.
						Time	Confidence range	
Broth B	6	6.7	(0.1)[b]	(4)	14[c]	14.9	10.8–20.7	Graham and
	10	6.7	(0.1)	(4)	3.6	6.4	5.0–8.4	Lund, 1993
	20	6.7	(0.1)	(4)	0.8	1.4	1.0–2.0	
Turkey	12	6.3	(0.2)	2	5	13.7	9.6–19.5	Meng and
B and E	12	6.3	(0.2)	4	3.2	4.9	3.8–6.4	Genigeorgis,
No typing	12	6.3	(1.2)	4	5	4.2	3.1–5.7	1993
	12	6.3	(0.2)	2	7	4.5	3.2–6.2	
Chicken	20	6.3	(1.5)	1	2	4.5	3.1–6.6	
	20	6.3	(1.5)	4	1	1.4	1.0–2.0	
	12	6.3	(1.5)	1	8	18.4	10.7–32.0	
	12	6.3	(1.5)	4	4	4.1	3.0–5.8	
Cooked meat, B, E and F	6	6.2	0.1	6[a] (5)	7.5	7.2	4.6–11.3	Peck et al.,
B toxin at 6°C, B, E and F at 12°C on day 14	12	6.2	0.1	6[a] (5)	2.5	2.5	1.6–4.0	1995
Broth	5	6.0	0.1	5	24.8[d]			Graham et al.,
B, E and F				4		23.2	16.5–32.9	1996
	10	6.0	4	5	26.9[d]			
				4		14.6	6.9–31.1	
	20	6.9	3	5	1.6[d]			
				4		1.7	1.1–2.8	

[a]Values are outside the range of the model, closest values allowed are used for calculations (in parentheses). ( ), Values are not given in the literature and are estimated.
[b]Personal communication, M. Peck, Norwich, UK.
[c]Calculated as 15 doubling times of vegetative cells, not from spores.
[d]Estimated from Barranyi model, lag plus 10 doublings from 10^3/ml.

4. Conclusion

The model proposed in this paper gives a good indication of the expected growth patterns of non-proteolytic type B *C. botulinum*. It demonstrates the marked effect spore numbers can have on the time for growth and subsequent toxin formation and indicates a need for additional research on the spore germination process in order to interpret laboratory data for commercial situations.

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